Suicide Inactivation of Monoamine Oxidase by 
trans-Phenylcyclopropylamine*

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The mechanism of the inactivation of monoamine oxidase by arylcyclopropylamines has been studied using [2-14C]DL-trans-phenylcyclopropylamine. This compound is a typical suicide inhibitor of bovine liver monoamine oxidase. It reacts rapidly and irreversibly with the enzyme, resulting in the incorporation of slightly over 1 mol of the inhibitor; the reaction is prevented but not reversed by substrates. Further, the enzyme oxidizes trans-phenylcyclopropylamine to the imine which, either as such or after hydrolysis to phenylcyclopropanone, combines irreversibly with the protein. Although inactivation is accompanied by bleaching of the 450 nm band of the covalently bound flavin component, which remains bleached even in the presence of air, the flavin ring system is not the site of combination of the inhibitor. This is shown by several lines of evidence. First, acid or heat denaturation releases all the 14C-labeled inhibitor and results in reoxidation of the flavin under conditions where adducts with N(5) or C(4a) of the flavin would be stable. Second, labeled inhibitor. It is suggested that the imine (or ketone) formed on oxidation of the inhibitor by the enzyme oxidizes trans-phenylcyclopropylamine to the imine which, either as such or after hydrolysis to phenylcyclopropanone, combines irreversibly with the protein. Although inactivation is accompanied by bleaching of the 450 nm band of the covalently bound flavin component, which remains bleached even in the presence of air, the flavin ring system is not the site of combination of the inhibitor. This is shown by several lines of evidence. First, acid or heat denaturation releases all the 14C-labeled inhibitor and results in reoxidation of the flavin under conditions where adducts with N(5) or C(4a) of the flavin would be stable. Second, following proteolysis at neutral pH and anaerobiosis, the 14C-labeled inhibitor may be separated chromatographically from the flavin peptide fraction. Third, on progressive denaturation the rate and extent of reoxidation of the flavin outstrips the dissociation of the labeled inhibitor. It is suggested that the imine (or ketone) formed on oxidation of the inhibitor by the flavin combines with an SH group at the substrate binding site to form a thioaminoketal or thiohemiketal, and this structure is stabilized by noncovalent interactions in the native enzyme but becomes labile and dissociable when the enzyme is unfolded. The fact that the flavin moiety of the enzyme remains reduced even in air may represent a steric hindrance to the access of oxygen to the reduced flavin by the bulky phenylcyclopropyl group.

Recognition of the cardinal role of monoamine oxidase in the catabolism of biogenic amines and of the usefulness of certain inhibitors of the enzyme in the treatment of mental depression has led to the development of a series of highly specific, irreversible inhibitors of the enzyme. The three main classes of active site-directed, irreversible inhibitors of mitochondrial monoamine oxidase are acetylenic amines, substituted hydrazines, and arylcyclopropylamines. Acetylenic amines and hydrazines are typical suicide inhibitors of the enzyme. The former act by forming a flavocyanine with N(5) of the covalently bound flavin component (1). Arylhydrazines are oxidized to the corresponding aryldiazene (2), which then inactivates the enzyme, at least in part, by reacting with C(4a) of the flavin (3). The purpose of the present paper is to show that arylcyclopropylamines are also suicide inhibitors but that their target is an amino acid component of the catalytic site, rather than the flavin.

EXPERIMENTAL PROCEDURES

Monoamine oxidase from beef liver was purified as previously described (4). Its concentration was estimated from the stoichiometric binding of [3H]parargyline (4). DL-trans-Phenylcyclopropylamine hydrochloride (PCPA) was obtained from the Sigma Chemical Co. [2-14C]Trans-Phenylcyclopropylamine sulfate, 0.69 Ci/mol, DL-trans-phenylcyclopropylamine, and L-trans-phenylcyclopropylamine were a kind gift of Drs. J. W. Wilson and C. Kaiser, Smith, Kline and French laboratories.

Monoamine oxidase activity was determined spectrophotometrically by the method of Tabor et al. (6) using benzylamine as substrate. Spectra were recorded with a Cary 14 instrument, interfaced with a Nova 2/4 computer. Other materials and methods were as in previous work (4).

RESULTS

Kinetics of Inactivation—The studies of McEwen et al. (6) clearly suggested that PCPA is an active site-directed, irreversible inhibitor of monoamine oxidase. One observation in disagreement with this was an earlier report (7) that dialysis against benzylamine partially reactivated the enzyme, following complete inhibition by PCPA. In our hands, neither dialysis against substrate nor gel exclusion on columns equilibrated with substrate resulted in any recovery of the activity. In fact, the data to be presented establish that PCPA is a typical suicide inhibitor of hepatic monoamine oxidase.

The progress of the inactivation at a low concentration of DL-PCPA is shown in Fig. 1. The reaction consists of two second order reactions. The apparent second order rate constant for inactivation for D-PCPA at 30°C and pH 7.2, derived from pseudo-first order kinetics was 5 x 10^3 M^-1 min^-1 (data not shown). From initial rate kinetics an apparent K_i of 4 µM was obtained. Since only the nonprotonated form of the inhibitor is active and its pK_a is 8.2 (6), the actual K_i of D-PCPA may be calculated (6) to be 0.36 µM. For L-PCPA the apparent second order rate constant for the inactivation was 6.5 x 10^3 M^-1 min^-1, the apparent K_i was 330 µM, and the K_i corrected for the proper ionizing form was 30 µM. The fast phase in Fig. 1, therefore, represents the inactivation of monoamine oxidase by the D-PCPA and the subsequent slow phase by the L-PCPA. The fact that inactivation did not reach

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* The abbreviation used is: PCPA, trans-phenylcyclopropylamine.
completion even in 1 h is due to the fact that the concentration of the enzyme exceeded that of PCPA. At higher ratios of inhibitor, enzyme loss of activity was complete in a few minutes and the reaction assumed pseudo-first order kinetic characteristics.

Absorbance Changes during Inactivation—The inactivation of monoamine oxidase is accompanied by complete bleaching of the absorbance of its flavin component in the visible, whether the experiment is carried out aerobically, as in Fig. 2, or anaerobically. The decline of absorbance at 450 nm is as calculated from the molar absorbance of FAD at this wavelength (10.3 × 10⁴ M⁻¹ cm⁻¹) and dithionite causes no further bleaching. The difference spectra shown in the inset are not entirely characteristic of flavin reduction, as is observed in addition to bleaching of the 450 nm band they also show a wide shoulder centering around 390 nm.

As expected of an active site-directed irreversible inhibitor, the extent of inactivation during the rapid phase varies linearly with the amount of inhibitor added and is paralleled by a loss of absorbance at 450 nm (Fig. 3). Complete bleaching of the flavin band at 450 nm and full inactivation are reached at a ratio of 1.1 to 1.2 mol of d-PCPA/mol of enzyme. At this point slightly over 1 mol of radioactive inhibitor is tightly bound to the enzyme, as may be ascertained by using [¹⁴C]-PCPA and removing uncombined inhibitor by centrifugation through columns of Sephadex G-25 (Fig. 4).

Oxidation of PCPA by Monoamine Oxidase: Evidence for Suicide Inhibition—If PCPA is a true suicide inhibitor, it should be oxidized by monoamine oxidase to a form which then combines very tightly with some component of the catalytic site, resulting in inactivation. While oxidation of PCPA to the imine or ketone seems a priori plausible, Hellerman and Erwin (7) concluded some years ago that this reaction does not occur. Their conclusion was based on the observation that, while prior reduction of monoamine oxidase with dithionite prevented inactivation of the enzyme in an-

![Fig. 1](image1.png)

**Fig. 1.** The rate of inactivation of monoamine oxidase by PCPA. The enzyme (0.4 μm) was incubated with 0.256 μm PCPA in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer containing 1% (w/v) Triton X-100, pH 7.2 at 30°C. At indicated times aliquots were withdrawn and their enzyme activity was compared to the uninhibited enzyme.

![Fig. 2](image2.png)

**Fig. 2.** Spectral changes accompanying the inhibition of monoamine oxidase by PCPA. The enzyme (11 μm) was incubated with 0.625 μm PCPA in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer containing 1% (w/v) Triton X-100, pH 7.2 at 30°C. At indicated times aliquots were withdrawn and their enzyme activity was compared to the uninhibited enzyme.

![Fig. 3](image3.png)

**Fig. 3.** Comparison of decrease of absorbance at 450 nm and loss of enzyme activity during the reaction of PCPA with monoamine oxidase. The enzyme (11 μm) was incubated with increasing concentrations of PCPA at 22°C in a buffer system described in the legend of Fig. 1. Spectra were taken after a 10-min incubation period against a reference cuvette containing everything except the enzyme.

![Fig. 4](image4.png)

**Fig. 4.** Stoichiometry of binding of [¹⁴C]PCPA to monoamine oxidase. An aliquot of the enzyme (150 μl, 5.6 μm) was incubated with increasing concentrations of [¹⁴C]PCPA in a total volume of 180 μl at 30°C for a period of 10 min. Aliquots of 150 μl were withdrawn at that time and unbound PCPA was separated from the enzyme-inhibitor complex by rapid gel filtration through Sephadex G-25. Protein, total counts, and enzyme activity were determined in aliquots of the eluate. The numbers given on the abscissa are for the Dl mixture. They have to be divided by 2 to obtain the quantity of the rapidly reacting D form.
aerobiosis by pargyline, an acetylenic suicide inhibitor (because the oxidized flavin is required for converting pargyline to the inhibitory form), reduction of the flavin by dithionite seemed to have no effect on the inactivation of the enzyme by PCPA.

This conclusion is contradicted by the following experiment. The enzyme (44 µm) was incubated with 130 µm [14C]PCPA for 10 min at room temperature. After exhaustive dialysis, the enzyme-inhibitor complex, containing 19,500 cpm, was precipitated with 6.6% (w/v) perchloric acid, containing 0.027 M 2,4-dinitrophenylhydrazine. The reaction mixture was extracted with ethyl acetate and aliquots were chromatographed on silica gel thin layer plates in benzene:methanol (8:2, v/v). The sample derived from the inhibited enzyme gave a single, yellow spot, clearly a 2,4-dinitrophenylhydrazone, which is probably 2-phenylcyclopropanone.

It is clear from this experiment that the radioactive inhibitor liberated from the inactivated enzyme reacts with 2,4-dinitrophenylhydrazine and is, therefore, a carbonyl. Thus, dehydrogenation of PCPA by the enzyme must be an integral part of the inactivation process. We suspect that contrary results of Hellerman and Erwin (7) were due to the rapid oxidation of PCPA to the imine during the assay, which was conducted aerobically.

Is PCPA a Flavin-Directed Inhibitor?—It has been proposed (7) that aryloclopropylamines combine with monoamine oxidase at the same site as arylhydrazines and acetylenic amines. Subsequent studies (1, 3) demonstrated that both of these two types of suicide inhibitor form covalent adducts with the flavin, the former at C(4a), the latter at N(5). Adduct formation at one of these positions would, of course, explain why the flavin remains bleached in the inactivated enzyme even in the presence of air. Nevertheless, as shown below, the flavin is not the combining site of the inhibitor in the case of PCPA.

The first bit of evidence supporting this conclusion came from the observation that acid, alkali, or heat denaturation liberates from the enzyme the radioactive product formed.

**TABLE I**

Release of inhibitor by denaturing agents as function of time

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inhibitor remaining bound</th>
<th>(8 \text{ M urea at } 30^\circ \text{C})</th>
<th>(8 \text{ M urea at } 22^\circ \text{C})</th>
<th>1% SDS at (30^\circ \text{C})</th>
</tr>
</thead>
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<tr>
<td>0.5</td>
<td>0.95</td>
<td>0.94</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>0.90</td>
<td>0.92</td>
<td></td>
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<tr>
<td>5</td>
<td>0.75</td>
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<td></td>
</tr>
<tr>
<td>10</td>
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<td>0.78</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
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<td>0.60</td>
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<tr>
<td>60</td>
<td>0.41</td>
<td>0.64</td>
<td>0.73</td>
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</tbody>
</table>

The enzyme (40 µm) was incubated with 200 µm PCPA aerobically (a) or anaerobically (b) in the buffer described in the legend of Fig. 1. After dialysis, aliquots of these solutions were mixed with 10 M urea in the same buffer to achieve a final concentration of 8 M urea. Change of absorbance at 450 nm was monitored with a Gilford spectrophotometer at 30°C. In the anaerobic sample air was admitted at the point indicated by the arrow.

**Fig. 5.** Change of absorbance at 450 nm of monoamine oxidase inhibited by PCPA in the presence of 8 M urea as function of time. The enzyme (50 µm) was incubated with 200 µm PCPA aerobically (a) or anaerobically (b) in the buffer described in the legend of Fig. 1. After dialysis, aliquots of these solutions were mixed with 10 M urea in the same buffer to achieve a final concentration of 8 M urea. Change of absorbance at 450 nm was monitored with a Gilford spectrophotometer at 30°C.

**Fig. 6.** Chromatography of a trypsin-chymotrypsin digest of monoamine oxidase, previously inhibited with [14C]PCPA, on Sephadex G-25. The preparation of the sample and chromatography on Sephadex G-25 (0.9 × 82 cm column) in 0.05 M phosphate buffer, pH 7.2, were conducted anaerobically. Fractions of 0.6 ml were collected and their radioactivity and flavin content were determined, the latter after performic acid oxidation.

from the inhibitor. Since C(4a) and N(5) adducts of flavins are unstable to light, oxygen, and/or acid pH (9), the effect of denaturing agents on the enzyme was studied in the absence of light, at neutral pH, in the presence and absence of oxygen. Denaturation with 80% (v/v) ethanol at 4°C left ~80% of the radioactive label attached to the protein, while urea and sodium dodecyl sulfate even at room temperature failed to liberate over 25 to 35% of the inhibitor. Table I summarizes the results of representative experiments.

It may be seen that 8 M urea and 1% (w/v) sodium dodecyl sulfate, at neutral pH, liberate only a fraction of the inhibitor and that this is a rather slow process even at 30°C. Comparison of Fig. 5 and Table I shows that aerobic incubation of the inactivated enzyme in 8 M urea at 30°C results in 50% reoxidation of the flavin in 3 min and complete reoxidation in about 30 min, while the dissociation of radioactive inhibitor at these times was only 18 and 45%, respectively. If the experiment is carried out anaerobically, the flavin remains bleached as the inhibitor is dissociated, but on admission of air immediate and
complete reoxidation of the flavin occurs, even though, at the time indicated by the arrow in Fig. 5, over 40% of the radioactive inhibitor was still attached to the enzyme (cf. Fig. 5 and Table I). Hence, on slow denaturation at neutral pH, conditions may be found when a substantial part of the inhibitor is still protein-bound but the flavin is nevertheless fully reoxidized. This is, of course, incompatible with the suggestion that the inhibitor is bound either at N(5) or at C(4a) of the flavin.

Further evidence that some component of the enzyme other than the flavin is the combining site of the inhibitor came from the experiment of Fig. 6. A sample of monoamine oxidase inactivated with [14C]PCPA, from which uncombined inhibitor had been removed, was digested with trypsin-chymotrypsin at neutral pH and chromatographed on a column of Sephadex G-25, the entire experiment being conducted under conditions where C(4a) and N(5) adducts are stable (anaerobiosis, neutral pH, absence of light). Assays of each fraction for cysteinyl flavin (4) and for radioactivity demonstrated a clear separation of the flavin peptide fraction from the labeled inhibitor. Moreover, upon exposure to air the flavin peptide fraction was rapidly reoxidized and showed a normal absorption spectrum and fluorescence characteristics. This experiment confirms that the combining site of the inhibitor is not the flavin but some amino acid component of the catalytic site. The probable identity of this site is analyzed under "Discussion."

DISCUSSION

At the outset of this investigation, on the basis of kinetic studies (6, 7), it appeared likely that arylcyclopropylamines, as exemplified by PCPA, are suicide inhibitors of monoamine oxidase, as has been demonstrated for the other two classes of potent irreversible inhibitors of the enzyme, arylhydrazines (3), and acetylenic amines (1). Two observations (7) seemed to contraindicate this hypothesis. One was that dialysis against substrates causes major reactivation, the other that the oxidized form of the enzyme is not required for the development of the inactivation, implying that oxidation of PCPA by the enzyme is not required for its action. As summarized above, clear separation of the flavin peptide fraction from the labeled inhibitor in our hands indicates that reoxidation occurred under any condition tested. Further, the oxidation of PCPA to 2-phenylcyclopropanone by the enzyme has now been experimentally demonstrated. Thus, arylcyclopropylamines do fulfill all the normal criteria of suicide inhibition.

Since the initial step in the development of the inactivation is clearly oxidation by the flavin, it is not surprising that the 450 nm absorption band of the flavin is bleached during this event. The fact that the flavin remains bleached in the inactivated enzyme in the presence of oxygen, in turn, implies either that the oxidized inhibitor combines at C(4a) or at N(5) in a covalent linkage or that the reoxidation of the flavin moiety in the inactivated enzyme is somehow prevented. Several observations in this paper rule out the first possibility. First, denaturation under conditions which are not known to cleave alkyl- or aryl-substituents from C(4a) or N(5) result in dissociation of the labeled inhibitor and reoxidation of the flavin. Second, in proteolytic digests of the inactivated enzyme the oxidized inhibitor and the flavin appear in different fractions. Third, under relatively mild conditions of denaturation reoxidation of the flavin occurs much faster and more extensively than the release of the labeled inhibitor. We conclude, therefore, that (a) the inhibitor is attached to an amino acid component of the catalytic site, not to the flavin, and (b) this attachment somehow prevents reoxidation of the protein-bound flavin by oxygen.

As to the identity of the amino acid residue which is the target of the oxidized inhibitor, Schiff's base formation with a primary —NH2 group may be ruled out since treatment with NaBH4 failed to prevent the release of the labeled inhibitor from the enzyme on drastic denaturation. A more likely possibility is the —SH group(s) known to be present at the substrate site since 1945 (10). The nature and function of the cysteine residues of the enzyme and their relation to catalytic activity have been extensively studied in several laboratories in recent years (11-14) and the presence of —SH group(s) at the substrate site seems well established. The reaction of either the imine to form a thioaminoketal or of the cyclopropanone to form a thiohemiketal, as visualized in Fig. 7 would explain essentially all the known characteristics of the inactivation of the enzyme by PCPA. First, thioaminoketals (and, by analogy, thiohemiketals) are dissociable compounds, but such adducts with proteins may be extensively stabilized by noncovalent interactions, so that dissociation is completely prevented. An example of this is the reaction of oxalacetate with a thiol group at the active site of succinyl dehydrogenase (15, 16). In the deactivated conformation of the enzyme the thiohemiketal is extremely tightly bound, so that no release of oxalacetate is possible but in the activated conformation oxalacetate may be quantitatively displaced by substrates and on acid denaturation of the enzyme all of the oxalacetate is released (16). The tight binding of PCPA to native monoamine oxidase and its complete removal on drastic denaturation of the enzyme represent an analogous behavior. It seems possible, even likely, that with the flavin juxtaposed to the substrate site, it must be for efficient electron transfer, stabilization may be provided by hydrophobic interaction between the aromatic nuclei of the flavin and of the inhibitor.

This interpretation, in turn, provides an explanation for the fact that the flavin remains bleached (i.e., reduced) in the inactivated enzyme even in the presence of oxygen, since the postulated π-π interaction between the benzene nuclei might prevent the access of oxygen to the flavin. On progressive denaturation, as observed with urea and sodium dodecyl sulfate, gradual unfolding of the protein would permit rapid and complete reoxidation of the flavin, followed by a slower dissociation of the inhibitor from the thiol group. Finally, the unusual difference spectrum of the inactivated enzyme in the 360 to 420 nm region (Fig. 2) may also be due to a perturbation of the flavin spectrum by an interaction of its benzene nucleus with that of the inhibitor.

It may be noted that, following the completion of this study, a paper by Abeles (17) appeared, which postulates, by analogy.
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with studies on aldehyde dehydrogenase, the structure shown in Fig. 7 for PCPA-inactivated monoamine oxidase. In contrast to the behavior of PCPA, alkylarylcyclopropylamines were reported by Silverman and Hoffman (18) to form labile adducts with monoamine oxidase, which have been suggested to involve N(5) of the flavin.

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Suicide inactivation of monoamine oxidase by trans-phenylcyclopropylamine.
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